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# Lymphocyte Maturation in the Human Thymus

## Relevance of Purine Nucleotide Metabolism for Intrathymic T Cell Function

H. J. SCHUURMAN, J. P. R. M. VAN LAARHOVEN, R. BROEKHUIZEN,  
G. Th. SPIERENBURG, P. BREKELMANS, C. G. FIGDOR,  
Ch. H. M. M. DE BRUYN & L. KATER

Division of Immunopathology, Departments of Internal Medicine and Pathology, University Hospital, Utrecht, Department of Human Genetics, University Hospital, Nijmegen, Department of Biophysics and Immunology, The Netherlands Cancer Institute, Amsterdam, The Netherlands

Schuurman, H.J., van Laarhoven, J.P.R.M., Broekhuizen, R., Spierenburg, G.Th., Brekelmans, P., Figdor, C.G., de Bruyn, Ch.H.M.M. & Kater, L. Lymphocyte Maturation in the Human Thymus. Relevance of Purine Nucleotide Metabolism for Intrathymic T Cell Function. *Scand. J. Immunol.* 18, 539–549, 1983.

The combination of centrifugal elutriation as an efficient and reproducible method to separate thymocytes by size, micromethods to assess purine interconversion enzymes, and assessment of purine (deoxy)nucleoside inhibition of mitogen responses enabled us to study purine metabolism at the intrathymic level. Out of six fractions, four (nos. 3–6), containing medium- and large-sized lymphocytes, showed a proliferative response after stimulation with phytohaemagglutinin (PHA). In fractions 1–6 the number of cells with an immature immunological phenotype gradually decreased, and cells with the phenotype of mature cells gradually increased. The enzyme activity ratio of adenosine deaminase to purine nucleoside phosphorylase gradually decreased from 21 in fraction 1 to 7 in the last fraction (blood T-cell value, 0.7). We conclude that this enzyme activity ratio is a useful marker for intrathymic T-cell maturation stages. In PHA-responsive cell fractions (3–6), the sensitivity to inhibition of the PHA response by (deoxy)adenosine and deoxyguanosine was inversely related to the enzyme activity ratio of ecto-5'-nucleotidase to deoxycytidine kinase. These findings are compatible with the hypothesis that intracellular concentrations of phosphorylated (deoxy)nucleosides are related to this inhibition. We conclude that the differences in purine metabolism among the various (mitogen-responsive) human thymocyte fractions are related to lymphoid cell function. Since the number of cells contributing to the enzyme activities and the number of cells contributing to the proliferative response (about 15% of unseparated cells) differ considerably, it is not possible to evaluate enzyme activities in unseparated thymocytes in terms of relationships between purine metabolism and lymphocyte function.

H. J. Schuurman, Ph.D., Division of Immunopathology, Department of Internal Medicine, University Hospital, 101 Catharijnesingel, 3511 GV Utrecht, The Netherlands

The lymphocyte compartment of the human thymus displays considerable diversity in the maturation stage of the cells [6, 31]. There is a relation between functional capacity, immunological phenotype, and tissue localization of the lymphocytes. In cell suspension this has been documented for thymocyte subpopulations obtained by agglutination with peanut agglutinin (PNA) and centrifugal elutriation (separation on the basis of cell size) [14, 26, 27].

A normal purine metabolism [4, 9, 17, 22] is necessary for proper functioning of lymphoid

cells. This has been documented by the causal relationships between immunodeficiency diseases and inherited deficiencies of enzymes involved in purine metabolism [9, 22]. T lymphocytes in various maturation stages show considerable differences in purine enzyme make-up [17]. Cohen *et al.* [12] have investigated the relation between this variation and lymphoid cell function. They have shown that thymocytes are more sensitive to inhibition of the phytohaemagglutinin (PHA) response by deoxyguanosine than mature T cells from blood or

tonsil. This difference in sensitivity could be explained by a more effective trapping of toxic deoxyguanosine triphosphate in thymocytes owing to a higher nucleoside phosphorylating capacity (deoxycytidine kinase (dCK) activity) and to a lower dephosphorylating capacity (ecto-5'-NT activity) [7, 12, 20, 25].

Thus far, the association between purine enzyme make-up and intrathymic T-cell maturation has received little attention. In man [20] and in the rat [4, 5] cortical thymocytes have a higher activity of adenosine deaminase (ADA) and a lower purine nucleoside phosphorylase (PNP) activity than relatively more mature cells in the medulla. On the basis of the variation in purine enzyme make-up in thymocyte subpopulations, Ma *et al.* [20] have suggested that the above-mentioned variation in sensitivity to inhibition of proliferative responses by (deoxy)nucleosides also applies at the intrathymic level.

The centrifugal elutriation method enables an efficient and reproducible separation, without affecting the functional capacity of the cell [14, 27]. We have previously described a radiochemical micromethod to assess purine enzyme activities with 1000 to 6000 cells per assay [18]. The combination of these two techniques enabled us to study purine metabolism at the intrathymic level. In this study we looked for the relation between enzyme make-up and (deoxy)nucleoside inhibition of the proliferative response after PHA stimulation.

## MATERIALS AND METHODS

*Thymocytes, thymocyte fractions and peripheral blood T cells.* Human thymuses were obtained from patients undergoing cardiac surgery (age range, 2½ months–4 years). Pieces of tissue were fixed in an absolute alcohol to formol to acetic acid ratio (75:20:5) and embedded in paraffin for histological examination. All thymuses included in this study showed a normal architecture. Thymocytes were harvested in suspension after the remaining tissue had been minced thoroughly and washed twice with Hepes (25 mM)-buffered RPMI-1640 (H-RPMI, Gibco, Grand Island, N.Y.). Centrifugal elutriation of the thymocytes into six fractions was performed as described [14, 27] with a Beckman J21C centrifuge equipped with a JE-6 elutriator rotor, modified to enable elutriation at a constant flow rate by variation of the rotor speed. Heparinized blood samples were obtained from the thymus donors and from apparently healthy control donors. Mononuclear cells were isolated by standard Ficoll–Hypaque ( $d = 1.077$  g/ml) density gradient centrifugation. T lymphocytes were

purified by rosetting with 2-aminoethyl-isothiuronium bromide hydrobromide (AET)-treated sheep erythrocytes (SRBC) followed by Ficoll–Hypaque density gradient centrifugation and isotonic lysis of the erythrocytes in the pellet fraction with an ammonium chloride buffer. The suspension obtained consisted of more than 95% cells rosetting with SRBC. Contamination by remaining SRBC was less than 0.5%. The viability of unseparated thymocytes, thymocyte fractions 1–6, and blood T cells exceeded 95% in all experiments (trypan blue dye exclusion test).

The immunological phenotype of cells in the various fractions was assessed by indirect immunofluorescence with mouse monoclonal antibodies [26] and with fluorescein isothiocyanate-labelled PNA [27].

*Purine interconversion enzyme activities.* ADA (EC 3.5.4.4) and PNP (EC 2.4.2.1) activities were assessed by the micromethod described by us previously [18]. For the determination of dCK (EC 2.7.1.74) [33] and adenylate kinase (AdKin; EC 2.7.4.3) [19] methods described elsewhere were applied after adapting the assays to our microconditions. dCK activity was determined to measure the (deoxy)nucleoside phosphorylating capacity, because in human lymphocytes this enzyme predominantly phosphorylates (deoxy)adenosine and deoxyguanosine [8]. Ecto-5'-NT (EC 3.1.3.5) activity was assayed essentially by the method of Edwards *et al.* [13]. The results of purine enzyme assays were expressed in nmol/10<sup>6</sup> cells·h.

*Lymphocyte stimulation with PHA.* Cultures (150 µl, 10<sup>5</sup> cells/culture) were set up in round-bottomed microtitre plates in RPMI-1640 with 20 mM bicarbonate supplemented with penicillin (100 U/ml), streptomycin (100 µg/ml), L-glutamine (2 mM), and 20% (v/v) heat-inactivated human AB serum for 90 h at 37°C in a humidified atmosphere with 5% CO<sub>2</sub>. In PHA stimulation cells were incubated with 16 µg PHA (HA 15, Wellcome, Beckenham, England). One microcurie (methyl-<sup>3</sup>H)-thymidine (5 Ci/mmol; Radiochemical Centre, Amersham, England) was added 16–18 h before harvest. The cells were harvested on glass-fibre filters with a semi-automatic harvester (Skatron, Lierbyen, Norway), and radioactivity was counted in toluene scintillator (Packard, Downers Grove, Ill.) containing 0.1 g/l 1,4-bis-(5-phenyloxazole-2-yl) benzene (POPOP) and 5 g/l 2,5-diphenyloxazole (PPO). All cultures were carried out in quadruplicate. To enable the comparison of thymocytes and blood T cells, the number of cells cultured was chosen to be between the optimal number of cells to measure PHA responsiveness in thymocytes (5×10<sup>5</sup> cells/culture [24, 26]) and in blood T cells (0.4×10<sup>5</sup> cells/culture). However, thymocyte responses were generally too low to enable reliable measurements in inhibition experiments. To promote thymocyte proliferation, the culture medium was supplemented with human thymic epithelial conditioned medium (HTECM) [24, 27] together with conditioned medium from PHA-stimulated blood mononuclear cells as source of interleukins [34]. The latter medium was obtained from two unrelated donors at a concentration of 10<sup>6</sup> cells/ml each with 10 µg PHA in the culture medium described above. After 2 days the cells were washed extensively and



then cultured for another 2 days; subsequently the supernatant was harvested. The conditioned media were added together to the lymphocyte stimulation tests, each in a final dilution of 1:30.

**Effect of (deoxy)nucleosides on PHA stimulation.** 2'-Deoxyguanosine, 2'-deoxyadenosine, and adenosine (Sigma Chemical Co., St Louis, Mo.) were added in various concentrations to PHA-stimulated cultures. Each dose-response experiment consisted of eight measurements with (deoxy)nucleoside (deoxyguanosine, range 1–3000  $\mu\text{M}$ ; (deoxy)adenosine, range 0.1–300  $\mu\text{M}$ ) and one measurement in the absence of (deoxy)nucleoside. The experiments with (deoxy)adenosine were performed in the presence of the ADA inhibitor 2'-deoxycoformycin (Pentostatin®, Warner Lambert-Parke Davis, Detroit, Mich., USA; 5  $\mu\text{M}$  final concentration). The 2'-deoxycoformycin solution was added to the cultures 20–30 min before the addition of (deoxy)adenosine. The (deoxy)nucleoside concentration at which the PHA response was 50% of the response without inhibitor was determined graphically from inhibition curves. This concentration is denoted the 50% inhibitory dose ( $\text{ID}_{50}$ ).

**Statistical analysis.** The Mann–Whitney U test was used, and the Spearman rank correlation coefficient ( $r_s$ ) was calculated in the correlation studies.

## RESULTS

*Purine interconversion enzyme activities in unseparated thymocytes, thymocyte subfractions, and peripheral blood T cells* (Table I, Fig. 1)

By centrifugal elutriation (separation by cell size) six fractions were obtained. In fractions 1 and 2, containing mainly small thymocytes (90–95%), about 80% of the thymocytes were recovered. Fractions 3–6 contained 6%, 5%, 2% and 2% of unseparated thymocytes, respectively. The contribution of small thymocytes in these fractions decreased from 55% in fraction 3 to 25% in fraction 6. About 50% of the cells in

fractions 3–6 consisted of medium-sized lymphocytes. The percentage of large lymphoblastoid cells increased from 1% in fraction 3 to 21% in fraction 6. The macrophage contribution was less than 1% in fractions 1–4, 2% in fraction 5 and 5% in fraction 6. Cells with the phenotype of cortical thymocytes decreased gradually in fractions 1–6; for example, the percentages OKT6-positive and PNA-binding cells were  $80 \pm 19\%$  and  $64 \pm 3\%$  in fraction 1, and  $30 \pm 8\%$  and  $25 \pm 4\%$ , respectively, in fraction 6 (mean  $\pm$  SEM, no. = 5). In unseparated thymocytes  $74 \pm 5\%$  OKT6-positive cells and  $55 \pm 4\%$  PNA-binding cells were found; on blood T cells these markers were hardly detectable ( $<0.5\%$ ). The percentage of cells with the phenotype of mature T cells gradually increased; HLA-ABC-bearing cells increased from  $10 \pm 2\%$  in fraction 1 to  $32 \pm 6\%$  in fraction 6 ( $13 \pm 3\%$  of unseparated thymocytes and almost 100% of blood T cells expressed this marker).

The ADA and dCK activities of unseparated thymocytes were significantly higher than those of blood T cells ( $P < 0.01$ ); the PNP, AdKin, and ecto-5'-NT activities were significantly lower ( $P < 0.01$ ) (Table I). In the thymocyte subfractions the lowest enzyme activities were observed in fraction 1. ADA, PNP, dCK and AdKin activities showed a gradual rise in fractions 1–6. The ecto-5'-NT activity was maximal in fraction 3, with a gradual decrease in the following fractions. None of the fractions showed the enzymatic make-up of blood T cells: only PNP and Adkin activities reached blood T cell values in fractions 5 and 6. Since the gradual rise in enzyme activities from fraction 1 to 6 (that is, with increasing cell size [14]) may be the

TABLE I. Activities of some purine interconversion enzymes in unseparated thymocytes, thymocyte subfractions, and blood T cells of the thymus donor\*

	Adenosine deaminase	Purine nucleoside phosphorylase	Ecto-5'- nucleotidase, $\times 10^3$	Deoxycytidine kinase, $\times 10^3$	Adenylate kinase
Unseparated thymocytes	$760 \pm 50$	$50 \pm 4$	$630 \pm 100$	$98 \pm 14$	$120 \pm 20$
Fraction 1	$730 \pm 90$	$38 \pm 6$	$350 \pm 70$	$82 \pm 11$	$85 \pm 17$
Fraction 2	$770 \pm 70$	$49 \pm 8$	$920 \pm 170$	$96 \pm 10$	$120 \pm 10$
Fraction 3	$970 \pm 100$	$83 \pm 11$	$1980 \pm 350$	$180 \pm 20$	$170 \pm 10$
Fraction 4	$1030 \pm 50$	$94 \pm 10$	$1290 \pm 300$	$240 \pm 30$	$190 \pm 20$
Fraction 5	$1330 \pm 50$	$140 \pm 20$	$910 \pm 290$	$390 \pm 30$	$270 \pm 10$
Fraction 6	$1150 \pm 80$	$180 \pm 30$	$600 \pm 230$	$340 \pm 40$	$340 \pm 50$
Blood T cells	$130 \pm 20$	$200 \pm 20$	$8570 \pm 980$	$53 \pm 9$	$260 \pm 80$

\* Values in nmol/ $10^6$  cells·h, mean  $\pm$  SEM of seven experiments.

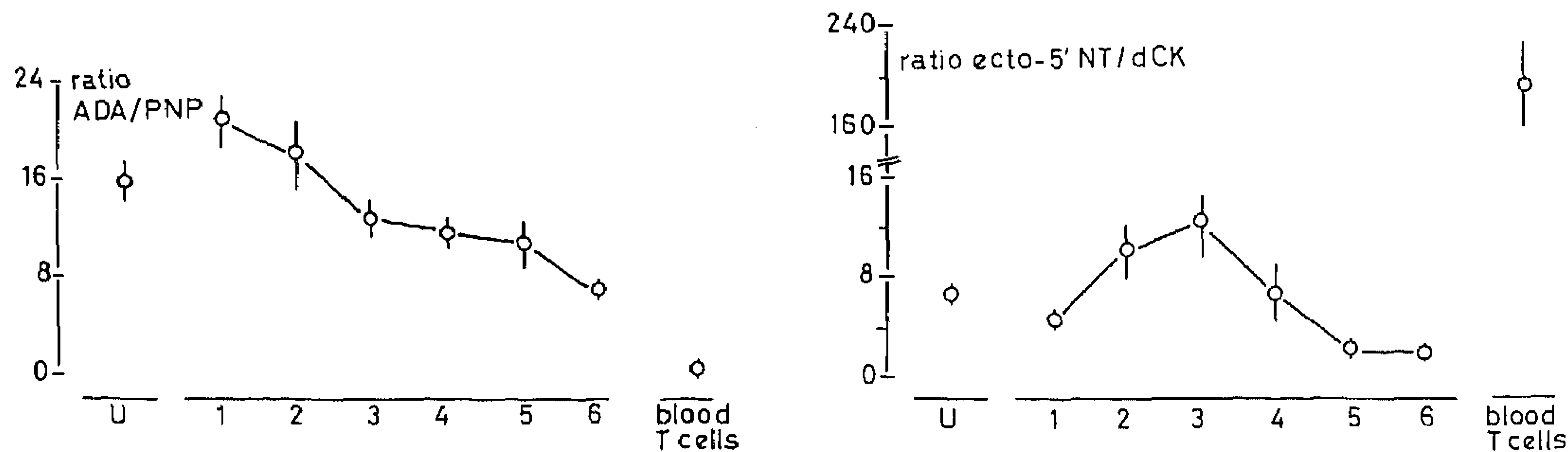


FIG. 1. Activity ratios of some purine interconversion enzymes in unseparated thymocytes (U), thymocyte fractions 1–6, and blood T cells of the thymus donor. ADA = adenosine deaminase; PNP = purine nucleoside phosphorylase; ecto-5'-NT = ecto-5'-nucleotidase; dCK = deoxycytidine kinase. Data are presented as the mean ratios  $\pm$  SEM of seven experiments.

reflection of increasing protein content, we expressed the results also as enzyme activity ratios (Fig. 1). The ADA/PNP enzyme activity ratio was calculated, since literature data indicate that this ratio is a marker of T-cell maturation [4, 22]. In unseparated thymocytes this ratio ( $16 \pm 2$ ) was significantly higher than in blood T cells ( $0.7 \pm 0.1$ ,  $P < 0.001$ ). In the thymocyte subfractions the ADA/PNP activity ratio decreased from 21 in fraction 1 to seven in fraction 6. Compared with unseparated thymocytes the ratio was significantly different in fractions 4 ( $P < 0.05$ ) and 5 and 6 ( $P < 0.01$ ).

Furthermore, we calculated the ecto-5'-NT/dCK enzyme activity ratio, since this ratio is related to the net capacity of the cell to convert (deoxy)nucleosides to toxic (deoxy)ribonucleotides (Fig. 1). This ratio was significantly lower in unseparated thymocytes ( $6.5 \pm 0.5$ ) than in blood T cells ( $190 \pm 40$ ,  $P < 0.001$ ). In thymocyte subfractions the ratio gradually increased from 4.4 in fraction 1 to 12 in fraction 3 and decreased in the subsequent fractions to 1.9 in fraction 6. This pattern paralleled that found for the ecto-5'-NT activity alone. The enzyme activity ratio of fraction 3 was significantly

TABLE II. Effect of conditioned media on the proliferative response of unstimulated and phytohaemagglutinin-stimulated cultures of unseparated thymocytes, thymocyte subfractions, and blood T cells\*

	Unstimulated cultures		Phytohaemagglutinin stimulation	
	Without conditioned media, cpm	With conditioned media, cpm	With conditioned media, cpm	Potentiating effect of conditioned media†
Unseparated thymocytes	360±80	1340±550	12,100±3500	4.6±0.9
Fraction 1	560±100	610±50	780±270	0.8±0.2
Fraction 2	400±120	650±30	880±250	1.4±0.3
Fraction 3	690±130	800±380	17,200±7300	3.2±1.3
Fraction 4	960±350	1030±720	14,200±6200	2.9±1.8
Fraction 5	1830±230	2150±600	22,900±5500	4.6±0.5
Fraction 6	1310±170	4560±2250	29,700±6600	17.5±2.8
Blood T cells	1010±250	3610±1250	42,100±3900	0.9±0.3

\* Conditioned media from cultured human thymic epithelial cells and from PHA-stimulated blood mononuclear cells were added, each in a final dilution of 1:30, to cultures of  $10^5$  cells in the presence or absence of  $16 \mu\text{g}$  PHA. Data are presented as mean values (counts per minute, cpm)  $\pm$  SEM of five experiments.

† The potentiating effect is calculated as the mean cpm in quadruplicate PHA-stimulated cultures in the presence of conditioned media, divided by the mean cpm in quadruplicate PHA-stimulated cultures without conditioned media.

different from that of fraction 4 ( $P < 0.05$ ) and of fractions 1, 5 and 6 ( $P < 0.01$ ). Compared with unseparated thymocytes, the ecto-5'-NT/dCK enzyme activity ratio was significantly different in fractions 1 ( $P < 0.02$ ), 3 ( $P < 0.05$ ), and 5 and 6 ( $P < 0.01$ ).

#### Lymphocyte stimulation with PHA (Table II)

The mean  $^3\text{H}$ -thymidine incorporation of unstimulated cells varied between 360 and 1830 counts per minute (cpm) in the various fractions. Addition of the conditioned media had a negligible effect on the response of unseparated thymocytes and subfractions 1–5, but a 3.5-fold enhancement of the response of fraction 6 and of blood T cells was observed. Thymocyte fractions 1 and 2 showed almost no response after PHA stimulation, and the potentiating effect of conditioned media on this response was minimal. A significant enhancing effect was observed in fractions 3–6. There was no potentiating effect of conditioned media on the PHA response of blood T cells: this observation is in agreement with our previous data on the biological effects of HTECM [24] and with the data on the independence of mature T cells from

exogenous growth factors in mitogen stimulation [34]. Since the PHA response of fractions 1 and 2 was negligible, these fractions could not be tested for (deoxy)nucleoside inhibition of the response.

#### Effect of (deoxy)nucleosides (Table III, Fig. 2)

Since the ADA activity of thymocytes was higher than that of blood T cells (Table I), it is conceivable that a higher concentration of deoxycoformycin is needed to obtain complete inhibition of ADA activity in thymocytes. We therefore studied the inhibition of the PHA response by (deoxy)adenosine with various concentrations of deoxycoformycin. The ADA activity in  $0.4 \times 10^5$  unseparated thymocytes and  $10^5$  blood T cells was completely inhibited at deoxycoformycin concentrations of  $1 \mu\text{M}$  or more, but deoxycoformycin concentrations of  $4 \mu\text{M}$  or more were needed for complete ADA inhibition in  $10^5$  unseparated thymocytes (data not shown). On the basis of these results we used a concentration of  $5 \mu\text{M}$  deoxycoformycin in the studies with (deoxy)adenosine. The presence of deoxycoformycin in this concentration had a

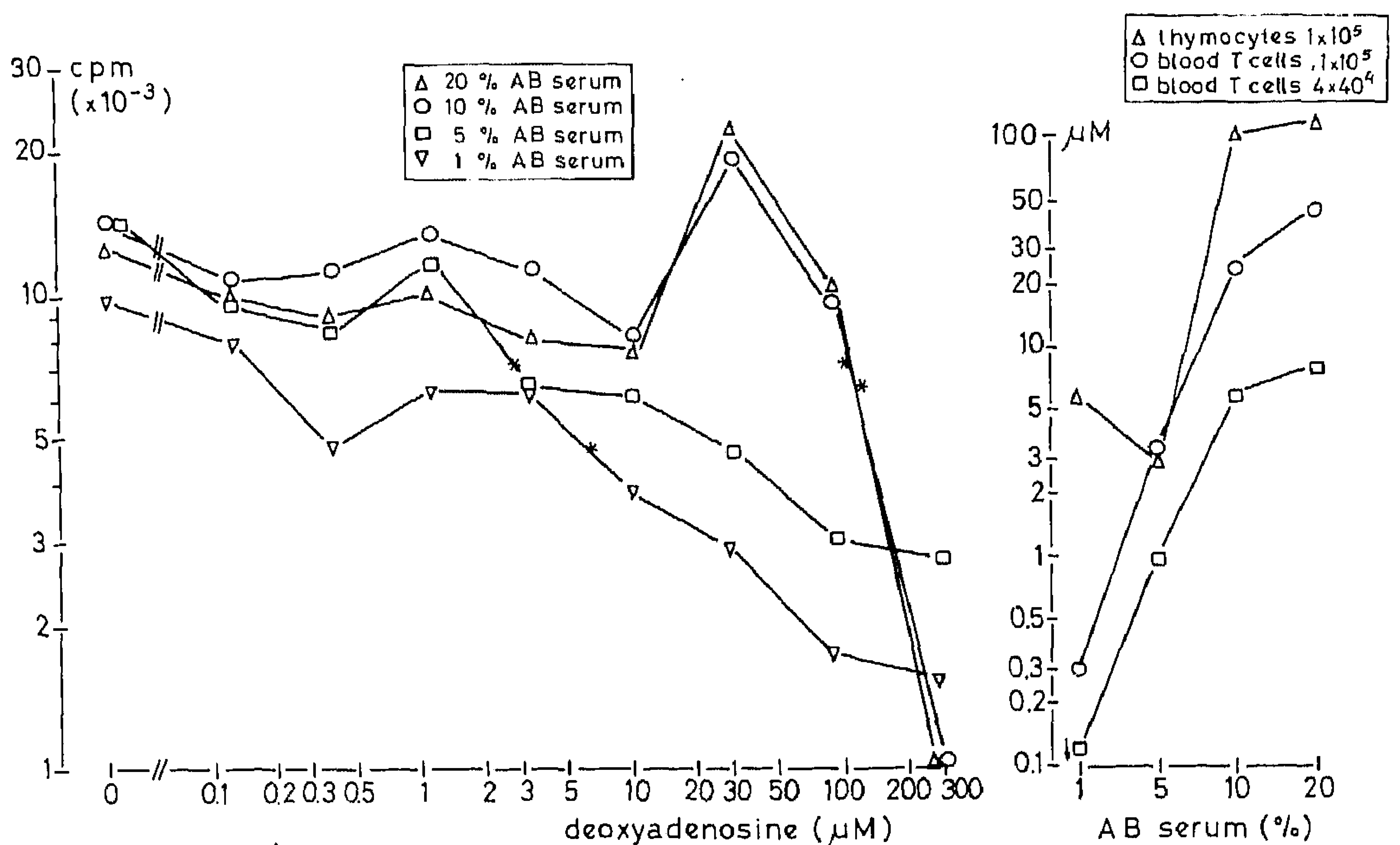


FIG. 2. Effect of concentration of AB serum in the culture medium on inhibition of the PHA response by deoxyadenosine. Left: inhibition curves (counts per minute versus concentration of deoxyadenosine) of the PHA response of unseparated thymocytes at various concentrations of AB serum; the 50% inhibitory doses of deoxyadenosine ( $\text{ID}_{50}$ ) are marked (\*). Right: dependence of  $\text{ID}_{50}$  (expressed in  $\mu\text{M}$  deoxyadenosine) in unseparated thymocytes and blood T cells on the AB serum concentration.



TABLE III. Effects of deoxycoformycin, (deoxy)adenosine, and deoxyguanosine on the phytohaemagglutinin response of unseparated thymocytes, thymocyte subfractions, and blood T cells\*

	Deoxycoformycin			
	Deoxycoformycin	Deoxyadenosine	Adenosine	Deoxyguanosine
Unseparated thymocytes	1.0±0.1	1.3±0.2	3.0±0.5	2.2±0.3
Fraction 1	1.2±0.1	1.8±0.3	3.2±0.7	2.0±0.4
Fraction 2	1.7±1.2	1.5±0.2	1.4±0.2	2.4±0.3
Fraction 3	0.9±0.1	1.3±0.2	2.5±1.0	1.8±0.4
Fraction 4	0.9±0.1	1.1±0.1	1.2±0.1	1.4±0.2
Fraction 5	1.0±0.1	1.0±0.1	1.0±0.1	1.1±0.1
Fraction 6	1.1±0.1	1.1±0.1	1.0±0.1	1.2±0.2
Blood T cells	1.0±0.1	1.3±0.3	1.0±0.1	1.1±0.1

\* Data are presented as the mean cpm in quadruplicate PHA-stimulated cultures in the presence of the compounds divided by the mean cpm in quadruplicate PHA-stimulated cultures without compounds. Deoxycoformycin was used in a final concentration of 5  $\mu\text{M}$ ; effects of (deoxy)adenosine were noted in the range 0.4–4  $\mu\text{M}$  and of deoxyguanosine in the range 10–100  $\mu\text{M}$ . Mean values  $\pm$  SEM of five experiments are presented.

negligible effect on the PHA response of the various cell fractions (Table III).

In the presence of various concentrations of (deoxy)nucleosides we generally found enhanced PHA responses at low concentrations and inhibition at higher concentrations (illustrated for deoxyadenosine in Fig. 2). In enhancing the PHA response, the most pronounced effects were found in unseparated thymocytes and in fractions 1–3 (Table III): no enhancing effects were observed in fractions 4–6 and in blood T cells. Adenosine showed the highest effect.

The concentration of serum in the culture medium influenced the  $\text{ID}_{50}$  values of (deoxy)nucleosides (illustrated for (deoxy)adenosine in Fig. 2). This phenomenon could not be explained by the presence of (deoxy)nucleoside-catabolizing enzymes in the serum, since the activities of ADA and PNP in the AB serum used were negligible. In addition, we incubated a 0.9 mM stock solution of (deoxy)adenosine and a 9.0 mM stock solution of deoxyguanosine in culture medium containing 20% AB serum for 4 days at 37°C. The concentrations of adenosine and deoxyguanosine remained unchanged and that of deoxyadenosine was 90% of the initial value (assessed by high-performance liquid chromatography [1]). On the basis of these results we used 20% AB serum in the culture. At this serum concentration the  $\text{ID}_{50}$  was affected least, and the inhibition curves showed the steepest slopes around 50% inhibition of the

PHA response (illustrated in Fig. 2). This leads to a higher accuracy in the determination of the  $\text{ID}_{50}$ . Furthermore, the PHA response of

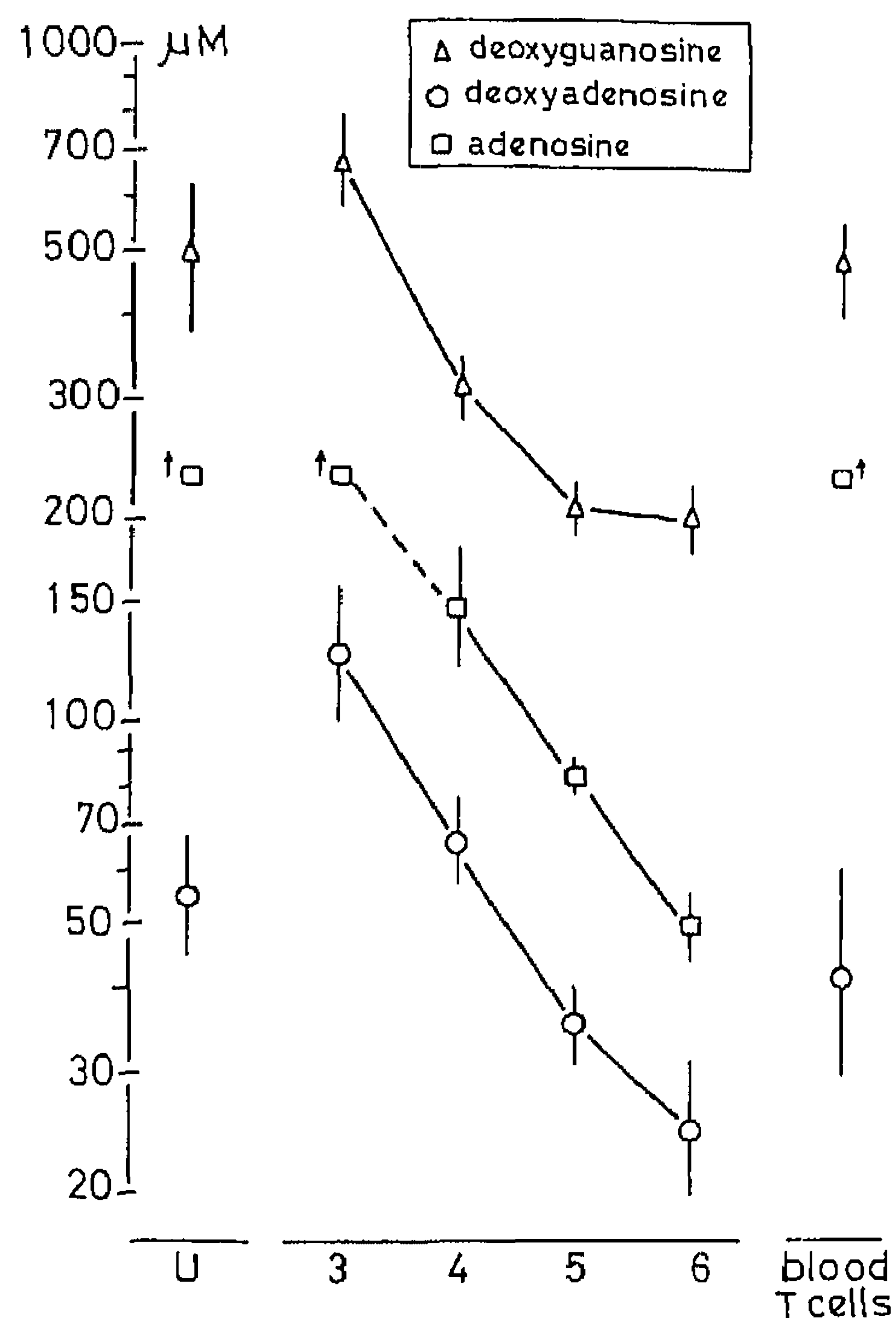


FIG. 3. Inhibition of the PHA response of unseparated thymocytes (U), thymocyte fractions 3–6, and blood T cells by (deoxy)nucleosides. Data presented are the geometric mean values of 50% inhibitory doses ( $\text{ID}_{50}$ , expressed in  $\mu\text{M}$  (deoxy)nucleoside)  $\pm$  SEM of four experiments.

thymocytes showed a large variability at serum concentrations of 10% or less (results not shown).

For all three (deoxy)nucleosides tested the  $ID_{50}$  was dependent on the number of cells cultured (illustrated for deoxyadenosine in Fig. 2). These findings prompted us to culture equal amounts of cells in all experiments to avoid this interference.

*Effect of (deoxy)nucleotides; relation to purine enzyme make-up (Figs. 3–5)*

Under the conditions defined in the above

experiments the  $ID_{50}$  of (deoxy)nucleosides in the PHA response of unseparated thymocytes, thymocyte subfractions 3–6, and blood T cells was determined (Fig. 3). In all fractions  $ID_{50}$  values were highest for deoxyguanosine, intermediate for adenosine, and lowest for deoxyadenosine. The differences in  $ID_{50}$  of (deoxy)nucleosides between unseparated thymocytes and blood T cells were not significant. The thymocyte subfractions showed a gradual decrease in  $ID_{50}$  from fraction 3 to 6. The differences between fraction 3 and fractions 4–6 were statistically significant ( $P$  varying between

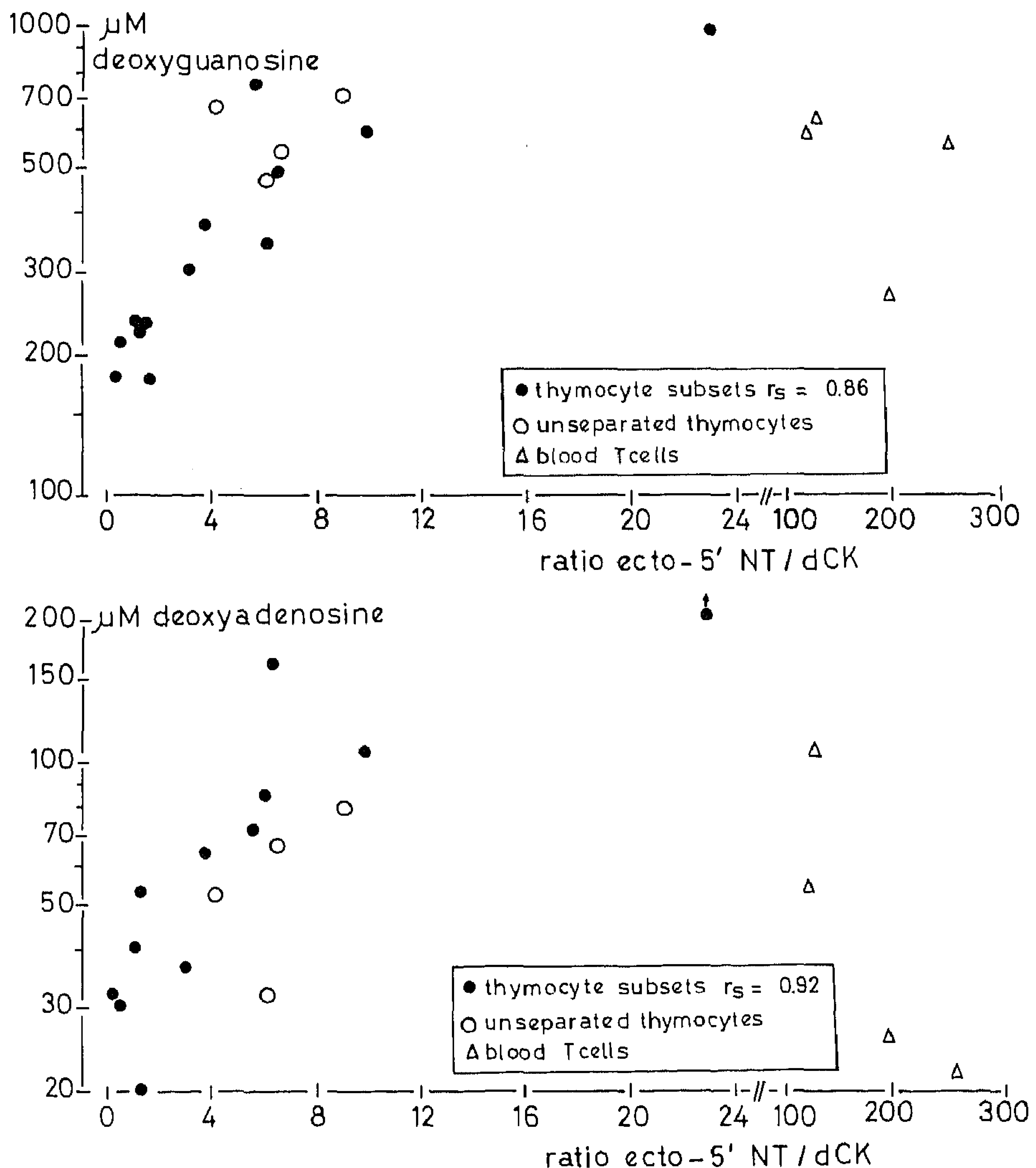


FIG. 4. Inhibition of the PHA response of unseparated thymocytes, thymocyte fractions 3–6, and blood T cells by deoxyguanosine and deoxyadenosine: correlation diagram of  $ID_{50}$  (expressed in  $\mu M$  deoxynucleoside) and ecto-5'-NT/dCK enzyme activity ratio.



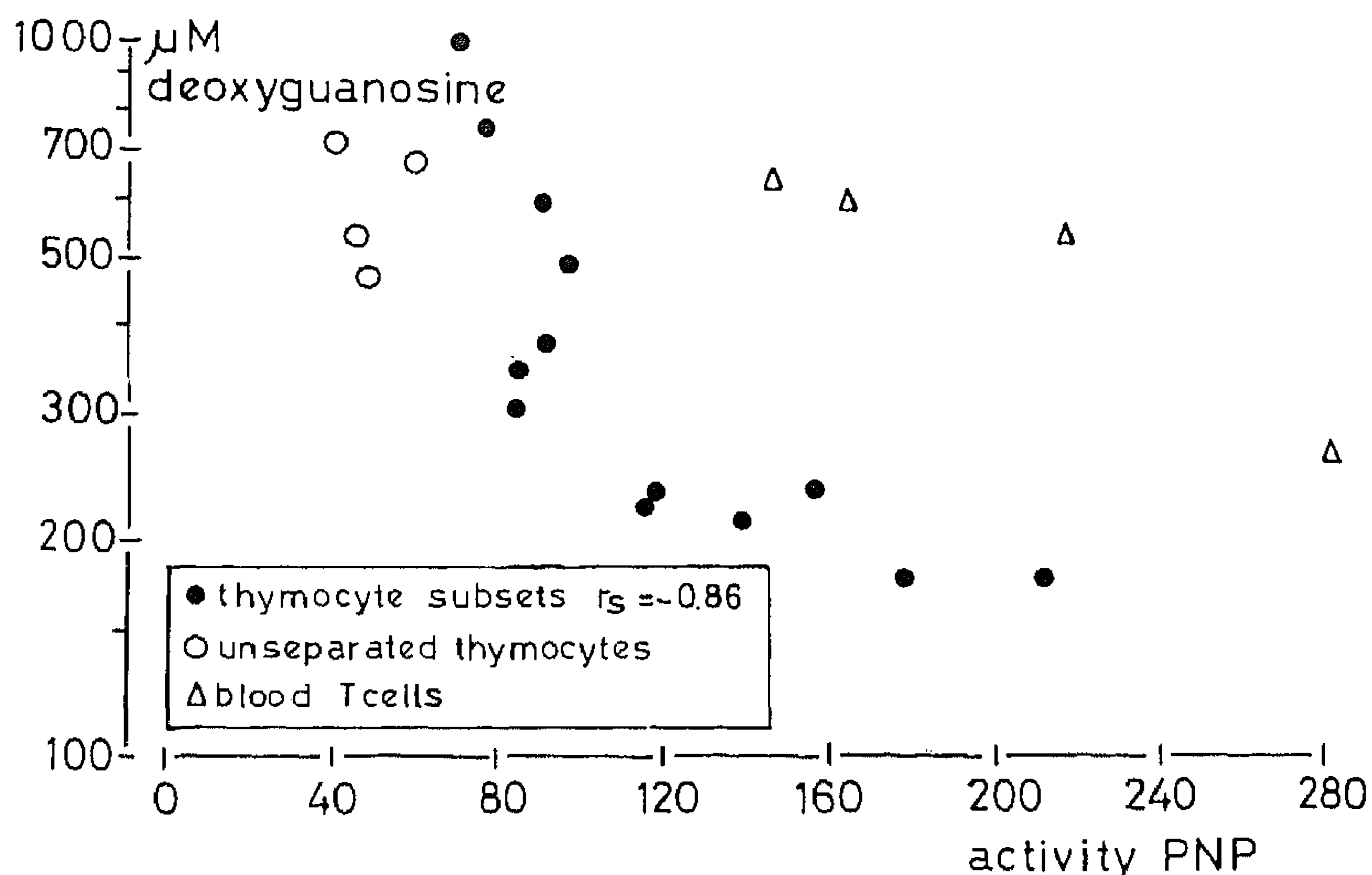


Fig. 5. Inhibition of the PHA response of unseparated thymocytes, thymocyte fractions 3–6 and blood T cells by deoxyguanosine: correlation diagram of ID<sub>50</sub> (expressed in  $\mu\text{M}$  deoxyguanosine) and PNP activity (expressed in  $\text{nmol}/10^6 \text{ cells}\cdot\text{h}$ ).

$<0.01$  and  $<0.03$ ). These differences apparently paralleled the difference in purine enzyme make-up (Table I, Fig. 1), especially that in the ecto-5'-NT/dCK enzyme activity ratio. Since the various cell fractions were simultaneously assessed for purine enzyme make-up and for (deoxy)nucleoside ID<sub>50</sub>, this parallelism could be analysed directly (Fig. 4). For the thymocyte subfractions the correlation coefficients of ID<sub>50</sub> values and the ecto-5'-NT/dCK enzyme activity ratio were 0.86 for deoxyguanosine, 0.92 for deoxyadenosine, and 0.87 for adenosine (data not shown). These correlation coefficients were statistically significant ( $P < 0.005$ ).

Unseparated thymocytes have been omitted from these calculations, since this population contains large amounts of PHA-unresponsive cells (present in fractions 1 and 2 after separation): these cells contribute to the enzyme activity but not to the ID<sub>50</sub>. A similar correlation between enzyme activity ratio and ID<sub>50</sub> seems to exist in unseparated thymocytes compared with thymocyte subfractions. The values of blood T cells were outside the range observed for unseparated thymocytes and thymocyte subfractions.

PNP activity was not inhibited under the present culture conditions, and conversion of deoxyguanosine to guanine is possible. Since guanine has been claimed to have a toxic capacity [21, 29], we correlated the ID<sub>50</sub> of deoxyguanosine with the PNP activity of the

various fractions (Fig. 5). In the thymocyte subfractions high PNP activities were associated with low ID<sub>50</sub> values ( $r_s = -0.86$ ,  $P < 0.005$ ).

## DISCUSSION

By means of centrifugal elutriation human thymocytes were separated into two large subsets of mitogen-unresponsive thymocytes (fractions 1 and 2) and four minor subsets containing mitogen-responsive cells. This difference in proliferative response between the subfractions was paralleled by differences in expression of immunological markers and, except for ecto-5'-NT activity, by differences in activities of enzymes involved in purine nucleotide metabolism (Table I, Fig. 1). Both in man [20] and in the rat [5] it has been shown that cortical thymocytes have a higher ADA activity than medullary cells; in addition, ADA-positive cells have been found exclusively in the thymus cortex by immunohistochemistry [10]. In contrast, we found lowest ADA activities in fractions 1 and 2 (Table I), which contain small cells localized in the cortex. The explanation of this discrepancy may be that the rise in absolute enzyme activities is the reflection of the increase in protein content (cell size). The effect of protein content is eliminated when the enzyme activity ratios are considered. We conclude that the ADA/PNP enzyme activity ratio is a useful

marker in human intrathymic T-cell maturation, which is in agreement with findings in the rat [5].

In the presence of *low concentrations* of (deoxy)nucleosides enhanced PHA responses were found (Table III). Agents that increase the intracellular cyclic AMP concentration induce maturation in immature thymocytes [16]. One of these agents, the thymus-dependent human serum factor [2], has been identified as adenosine [3]. Increased levels of cyclic GMP have been found in mouse thymocytes after incubation with thymosin fraction 5 [23]. The low concentrations of (deoxy)nucleosides used in our study may give rise to increased levels of intracellular cyclic AMP and cyclic GMP. Since the effects on the PHA response were most pronounced in unseparated thymocytes and in subfractions 1–3, we suggest that purine nucleosides, especially adenosine, are involved in induction of mitogen responsiveness in unresponsive thymocytes.

In the study of the inhibition of the PHA response at relatively *high concentrations* of (deoxy)nucleosides, the  $ID_{50}$  values of adenosine were higher than those of deoxyadenosine (Fig. 3). This observation is in agreement with literature data [15, 28, 32]. The variant toxicity of both compounds may be related to the different mechanisms involved in purine nucleoside-induced toxicity in lymphoid cells [4, 9, 22].

In thymocyte subfractions 3–6 an increased sensitivity to (deoxy)nucleoside inhibition of the PHA response was found (Fig. 3). The accumulation of toxic (deoxy)ribonucleotides in lymphoid cells induced by (deoxy)nucleosides has been reported [7, 12, 29, 33]. The concentrations of the (deoxy)nucleotides are positively affected by the phosphorylating capacity (dCK activity) and negatively affected by the dephosphorylating capacity (ecto-5'-NT activity) of the cell. We found significant correlations between the sensitivity to inhibition of the PHA response by (deoxy)nucleosides and the ecto-5'-NT/dCK enzyme activity ratio (Fig. 4). These correlations suggest that the intracellular concentrations of phosphorylated (deoxy)nucleosides are related to the inhibition of the proliferative response.

The correlation between  $ID_{50}$  values and the ecto-5'-NT/dCK enzyme activity ratio in unseparated thymocytes fits closely with the significant correlation found in the thymocyte subfractions (Fig. 4). This is merely the conse-

quence of the similar ecto-5'-NT/dCK activity ratio in fractions 1 and 2 (which do not contribute to the PHA response) when compared with fractions 3–6. The correlation of nucleoside-induced inhibition of the PHA response and purine enzyme make-up in unseparated thymocytes can lead to misleading conclusions, because the mitogen response of only a small subset (fractions 3–6 in the present study) is compared with the enzyme make-up of all cells.

The  $ID_{50}$  values of (deoxy)nucleosides in unseparated thymocytes were in the same range as those in blood T cells—that is, about  $500\ \mu\text{M}$  for deoxyguanosine. Cohen *et al.* [12] have reported a deoxyguanosine  $ID_{50}$  of about  $30\ \mu\text{M}$  in unseparated thymocytes and about  $150\ \mu\text{M}$  in blood mononuclear cells. These discrepancies can be explained in three ways:

First, differences existed in the methods of the experiments. In the study by Cohen *et al.* [12]  $0.5 \times 10^5$  cells were cultured in medium supplemented with 1% fetal calf serum, whereas in our study  $10^5$  cells were cultured in medium supplemented with 20% human AB serum. Both the number of cells cultured and the percentage of serum present in the culture medium affect the  $ID_{50}$  values (Fig. 2).

Second, in contrast to Cohen *et al.* [12] cultures were supplemented with conditioned media from human thymic epithelial cells and from PHA-stimulated blood mononuclear cells. The second medium served as a source of interleukins (T-cell growth factors) [34]. It has been reported that rat thymocytes fail to produce interleukins after mitogen stimulation in the presence of deoxyadenosine when ADA activity is inhibited [30]; the low mitogen response under these conditions was partially restored by the addition of exogenous interleukins. If this mechanism of inhibition of interleukin production by (deoxy)nucleosides holds for human thymocytes, it can explain the different  $ID_{50}$  values of deoxynucleosides in our study in the presence of interleukins. Similarly, the addition of HTECM may influence the  $ID_{50}$  determinations. A two- to threefold increase in ecto-5'-NT activity has been found in human thymocytes after incubation with this medium [11]. This change in enzyme activity has not been confirmed for the activities of ADA, PNP and ecto-5'-NT after incubation of thymocytes with thymic humoral factors [25]. If induction of



ecto-5'-NT by HTECM occurs under the conditions of our experiments, the subsequent higher dephosphorylating capacity of the cell leads to lower intracellular (deoxy)nucleotide concentrations and hence to higher ID<sub>50</sub> values of (deoxy)nucleosides.

Third, we found an inverse correlation between PNP activity and deoxyguanosine ID<sub>50</sub> values (Fig. 5). This may represent a causal relationship, since guanine, the product of PNP-mediated breakdown of deoxyguanosine, is toxic to the cell [21, 29]. This possible pathway in deoxyguanosine toxicity has not been considered by Cohen *et al.* [12]. We are not aware of effects of the culture conditions on the relative contribution of the various mechanisms of (deoxy)nucleoside-mediated toxicity.

We conclude that the culture conditions in lymphocyte stimulation affect the outcome of (deoxy)nucleoside intoxication studies and hence the interpretation of the results in terms of causal relationships between lymphoid cell function and purine enzyme make-up.

In this study we found that differences in purine enzyme make-up between thymocyte subfractions are related to lymphoid cell function. The variant (deoxy)nucleoside toxicity known in T lymphocytes in different stages of maturation [12] applies also at the intrathymic level.

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